

# Study of establishing disease-syndrome combined with animal model for immune thrombocytopenic purpura without additional conditions

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## KEYWORDS

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Disease-syndrome  
combined animal  
model

**Abstract** *Objective:* To explore the feasibility of establishing the disease-syndrome combined animal model for immune thrombocytopenic purpura (ITP) without additional conditions.

*Methods:* Three batches of data related to the ITP model mice obtained by replication at different time were analyzed, and whether the APS-injected model mice replicated through the passive immune modeling method could simulate the pathogenesis and clinical characteristics of human ITP was evaluated according to the differentiation criteria for disease-syndrome combined model.

*Results:* The APS-injected replicated ITP model mice possessed the following traits: (1) Compared with the normal group, the platelet count was significantly decreased, and coagulation time was significantly increased in the model group ( $P < .01$ ). (2) Compared with the normal group, the medullary thrombocytogenous megakaryocytes were significantly decreased ( $P < .05, .01, .001$ ). (3) The APS-injected sites and other parts of the model mice had spontaneous hemorrhage. (4) Behavioral changing signs were observed 1 week after the modeling (i.e. low activity, delayed activity, poor appetite, skin petechia/hemorrhage and spontaneous hemorrhage at the injected sites or other parts), and were getting more and more severe.

*Conclusion:* According to the syndrome differentiation criteria for disease-syndrome combined model of ITP, the APS-injected animal model of ITP replicated through the passive

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immune modeling method without additional conditions possesses the characteristics of disease-syndrome combined model. It provides an ideal tool for the development of traditional Chinese medicine pharmacology experiment.

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## Introduction

Disease-syndrome combination is an important diagnosis/treatment mode in traditional Chinese medicine (TCM), which is characterized by stressing on differentiation of syndromes and treatment.<sup>1</sup> In order to explore the action mechanism of medicinal herbs, the corresponding animal model should generally be established for verification. However, four diagnostic methods of TCM cannot be fully utilized on animal models (e.g. inquiry, pulse-taking and tongue inspection), thus the replicated animal model is basically just a disease model without any TCM syndromes.<sup>2</sup> To solve the problem, it is necessary to prepare a disease-syndrome or syndrome-disease model with other interventions based on chronic disease.

We hold the opinion that there is a gap between this model and disease occurrence and progress, for which the following idea is raised, i.e. now that a disease model can be replicated, it is possible to find out the TCM syndrome similar to human disease by observing some external signs of the model mice.<sup>3</sup> Based on this hypothesis, we made an overall analysis of the three batches of data related to the ITP model mice replicated without additional intervention, and evaluated whether the duplicated APS-injected ITP model mice prepared with the passive immune modeling method could simulate the occurrence and progress of disease and also clinical manifestations in human ITP according to the differentiation criteria for disease-syndrome combined model of ITP.

## Differentiation criteria for disease-syndrome combined model

### Identification criteria for disease model

The disease model of ITP mice must meet the following 4 criteria: (1) decrease in peripheral blood platelet count; (2) hemorrhage at the injection site or spontaneous skin petechial; (3) prolongation of blood coagulation time; (4) decrease in medullary thrombocytogenous megakaryocytes.

### Identification criteria for the syndrome model

According to human ITP clinical characters associated with the behavior features of the experimental mice, and some quantitative determination indices, the external manifestations of human "failure of qi to control blood" syndrome were converted to that of "failure of qi to control blood" syndrome of the laboratory animals. The conversion indexes included: (1) hemorrhage at the injection site and

other areas or spontaneous skin petechia (dim spot), which was the common features of the disease-syndrome; (2) decreased activity, similar to fatigue; (3) sluggishness, similar to limb weakness; (4) poor appetite, less intake of food and water, and loose stools, similar to spleen qi deficiency; (5) weight loss, similar to deficiency in the spleen which fails to control muscles.

## Differentiation criteria for the disease-syndrome combined model

According to the evaluation indices for the disease-syndrome model, the differentiation criteria for the disease-syndrome combined model of ITP was specified as follows: more than 3 of the above 4 indices observed in the disease model (note: Index 1 was compulsory) associated with any 3 of the above 5 indices.

## Methods

### Preparation of anti-mice-platelet serum

The anti-mice-platelet serum was prepared through the following procedures: (1) The BALB/c mice were taken, anesthetized with ether, and the whole blood from the mouse heart was drawn and anti-coagulated with EDTA-Na<sub>2</sub>. The platelet was separated, washed up and diluted with physiological saline. (2) The separated platelet was taken, mixed with an equivalent amount of complete Freund's adjuvant and incomplete Freund's adjuvant respectively into a water-in-oil form as the antigen. At week 0, the antigen of complete Freund's adjuvant was injected into at least 4 sites on the sole, back and hypodermis of the *Cavia porcellus*. On week 1, 2 and 4, the antigen of incomplete Freund's adjuvant was injected into the above 4 sites. On week 5, the non-anticoagulatory whole blood was drawn from the heart of the guinea-pig. After 560 g × 10 min centrifuging, the supernatant was taken and the *C. porcellus* anti-mice-platelet serum (GP-APS) was obtained, and stored in a -20°C refrigerator for later use. (3) Improvement was done by referring to the ELISA method. The potency of anti-platelet-serum was determined (i.e. replacing the pure product of China-made lyophilized ELISA A protein by the antibody marked with alkaline phosphatase-protein A enzyme). (4) The APS was taken out from a -20°C refrigerator, and placed in a 56°C water bath for 30 minutes. Absorption was carried out for at least twice with an equal amount of BALB/c mice erythrocyte, which was diluted into APS solution (1:4) with physiological saline for later use.

Grouping of laboratory animal

Healthy BALB/c mice (SPF-grade), 18–22 g, 8 weeks old, half male and half female were enrolled in our study. Before test, the blood was drawn via the caudal vein of 24 mice, and the peripheral platelet count was determined through the fully-automatic blood cell counter. The 24 mice were randomly divided into 2 groups (12 mice in each group). In the normal group, the mice were fed in a routine way. In the model group, on days 1, 3, 5, 7, 9, 11 and 13, APS solution (1:4) was injected intraperitoneally at 100  $\mu$ L/20 g for modeling; on day 15, after the dynamic observation of various indices, the mice were sacrificed, and the specimen was collected for index determination.

Determination methods for observation of indices

Main indices of observation as follows: (1) Disease model indices: platelet count, bleeding degree, coagulation time, and type of medullary megakaryocyte. (2) Syndrome model indices: dynamic observation of the behavior of the model mice and weight change.

Statistical methods

The data was expressed as mean  $\pm$  SD ( $\bar{x} \pm s$ ). The independent-sample *t*-test and one-way ANOVA were made through the SPSS software. *P* < .05 indicated a statistical significance.

Results

Change of the observation indices in the disease model

Platelet count

After the experiment, 80  $\mu$ L blood was drawn from the caudal vein and it was put into an anticoagulation tube with EDTA–Na<sub>2</sub>. After they were mixed well, blood count was determined by automatic cell counter. The results of platelet count of three test batches are shown in Table 1.

As shown in Table 1, after the modeling, the peripheral blood platelet count of model mice dropped obviously, which was statistically significant compared with those in the normal group of the same test batch (*P* < .01).

Table 1 Change in platelet count ( $\bar{x} \pm s$ ).

Test batch	Platelet count (10 <sup>9</sup> /L)	
	Normal group ( <i>n</i> )	Control group ( <i>n</i> )
1st test batch	967.00 $\pm$ 301.30 (12)	618.1 $\pm$ 192.10* (11)
2nd test batch	542.80 $\pm$ 116.13 (10)	313.60 $\pm$ 77.69* (10)
3rd test batch	552.00 $\pm$ 114.00 (9)	314.00 $\pm$ 78.00* (10)

Note: \**P* < .01, as compared with that in the normal group.

Hemorrhage degree

As shown in Figs. 1–3, in the continuous dynamic observation during the test, on day 3 after the injection of APS, a dense scarlet bleeding point appeared in the hypodermis, and the subcutaneous petechia merged into flake and then lasted for about 3–4 days. One week after the modeling, hemorrhage was gradually relieved, and the blood color gradually turned from scarlet to dark red, which was similar to the manifestations of chronic hemorrhage in human ITP. The bleeding degree of the three batches of mice is shown in Figs. 1–3.

Coagulation time

After tests, a drop of eye ball blood was taken and put into a vessel, and then disturbed with a syringe needle until blood streak appeared. The lasting time (second) was recorded. The results of coagulation time of the three test batches are shown in Table 2.



Figure 1 The first experiment showed bleeding in the abdomen and limbs.

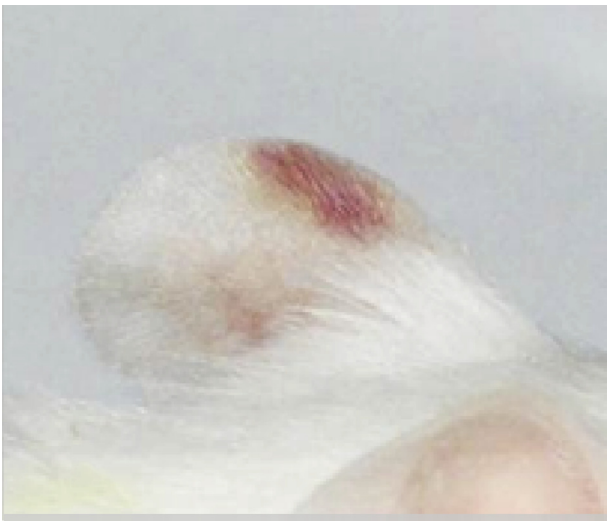


Figure 2 The second experiment showed bleeding in the ear.



**Figure 3** The third experiment showed bleeding in toes, and swelling.

**Table 2** Change in coagulation time ( $\bar{x} \pm s$ ).

Test batch	Coagulation time (s)	
	Normal group (n)	Control group (n)
1st test batch	62.66 $\pm$ 6.71 (12)	90.81 $\pm$ 8.70* (11)
2nd test batch	61.60 $\pm$ 2.88 (10)	81.90 $\pm$ 4.12* (10)
3rd test batch	61.60 $\pm$ 2.88 (10)	81.90 $\pm$ 4.12* (10)

Note: \* $P < .01$ , as compared with that in the normal group.

As shown in Table 2, after the completion of the test, the coagulation time of model mice rose, which was statistically significant compared with those in the normal group of the same test batch ( $P < .01$ ).

#### Change in the type of medullary megakaryocyte

After the tests the mice were sacrificed. The sternum marrow was taken and mixed well in 25  $\mu$ L calf serum. Smear samples were quickly prepared. Wright's staining after air dried. One hundred megakaryocytes were calculated in order and classified (megakaryoblast, promegakaryocyte, granular megakaryocyte and thrombocyctogenous megakaryocyte) by a microscope (10  $\times$  100). The results of medullary megakaryocyte of the three test batches are shown in Table 3.

**Table 3** Type of medullary megakaryocyte ( $\bar{x} \pm s$ ).

Test batch	Group (n)	Megakaryoblast	Promegakaryocyte	Granular megakaryocyte	Thrombocyctogenous megakaryocyte
1st test batch	Normal group (12)	6.25 $\pm$ 1.48	27.41 $\pm$ 5.05	58.16 $\pm$ 5.02	8.16 $\pm$ 2.25
	Model group (11)	4.18 $\pm$ 1.33	30.63 $\pm$ 4.50	60.00 $\pm$ 3.66	5.18 $\pm$ 1.83*
2nd test batch	Normal group (10)	28.90 $\pm$ 5.22	6.30 $\pm$ 2.45	56.80 $\pm$ 5.77	8.10 $\pm$ 1.85
	Model group (10)	32.20 $\pm$ 5.90	3.70 $\pm$ 1.42	58.9 $\pm$ 5.49	5.20 $\pm$ 1.48
3rd test batch	Normal group (10)	28.90 $\pm$ 5.22	6.30 $\pm$ 2.45	56.80 $\pm$ 5.77	8.10 $\pm$ 1.85
	Model group (10)	32.20 $\pm$ 5.90	3.70 $\pm$ 1.42	58.90 $\pm$ 5.49	5.20 $\pm$ 1.48

Note: \* $P < .05$ ,  $P < .01$ ,  $P < .001$ , as compared with that in the normal group.

As shown in Table 3, compared with those in the normal group of the same test batch, the count of medullary thrombocyctogenous megakaryocyte in the model mice dropped even more ( $P < .01$ ), and the count of medullary naked nucleus megakaryocyte in the model mice also dropped in the 2nd and 3rd test batches ( $P < .01$ ).

### Judgment of the syndrome model

#### Behavioral observation

In the normal group, there were flexible response, smooth hair, voluntary activity, active food rooting and stripy stool. In the model group, 1 week after the injection of APS, there were low activity, delayed activity, poor appetite, decrease in food/water intake and skin petechia/ecchymosis; and 2 weeks after the injection of APS, there were delayed response and withered hair, which was exacerbated with the modeling duration.

#### Weight

The results of mice weight of the 1st and 2nd test batches in both groups are shown in Table 4.

Table 4 showed that after the modeling of the 1st, 2nd and 3rd batches there was no difference of weight between the two groups in the 1st week. But from the 2nd week, the weight of the model group dropped obviously, and there was statistical significance in comparison with the normal group before modeling ( $P < .05$ ).

### Judgment of the disease-syndrome combined model

According to the above test results, besides the skin spontaneous hemorrhage at the injected sites or other parts, the mice model of ITP possessed the following manifestations: decrease in peripheral platelet count, increase in coagulation time, decrease in medullary thrombocyctogenous megakaryocyte, low activity, delayed activity, poor appetite, loose stool, lusterless hair, skin petechia, and weight loss. As judged according to the differentiation criteria for disease-syndrome combined model of ITP, the APS-injected animal model of ITP replicated through the passive immune modeling method possessed the characteristics of disease-syndrome combined model.



**Table 4** Comparison of mice weight g, ( $\bar{x} \pm s$ ).

Test batch	Group (n)	Before the modeling	1 week after the modeling	2 weeks after the modeling
1st test batch	Normal group (12)	18.63 $\pm$ 2.33	18.95 $\pm$ 1.14	21.17 $\pm$ 1.81
	Model group (11)	20.21 $\pm$ 1.51	17.69 $\pm$ 1.52	17.18 $\pm$ 1.94*
2nd test batch	Normal group (10)	20.10 $\pm$ 0.74	21.30 $\pm$ 0.82	21.80 $\pm$ 0.79
	Model group (10)	20.00 $\pm$ 0.82	19.50 $\pm$ 0.67	18.70 $\pm$ 0.95*
3rd test batch	Normal group (20)	24.030 $\pm$ 2.52	23.35 $\pm$ 2.16	21.00 $\pm$ 1.38
	Model group (20)	23.93 $\pm$ 2.66	22.07 $\pm$ 1.64	20.65 $\pm$ 1.35*

Note: \* $P < .05$ , as compared with that in normal group and before the modeling.

## Discussion

According to the severity of disease, ITP is classified into acute ITP (AITP) and chronic ITP (CITP).<sup>4</sup> Based on the TCM theory and the clinical manifestations, AITP often occurs in children, and is a syndrome of bleeding due to blood-heat. With acute pathogenesis, serious thrombocytopenia and massive hemorrhage, the following emergency measures are necessary to rapidly control the illness of AITP: platelet transfusion, hemostasis, and massive administration of adrenocortical hormone or immune globulin. For CITP, there are two types of syndrome: syndrome of failure of the spleen to control blood and syndrome of static blood blocking collateral.<sup>5</sup> In the long course of medical practice we have found traditional Chinese medicine is the most effective to CITP because it can relieve symptoms and bleeding, and improve peripheral platelet count.<sup>6</sup> But we still have to confirm whether a set of medicinal herbs used according to the theory of traditional Chinese medicine is effective and what the mechanism is. It is necessary to establish a disease-syndrome combined animal model of good stability and reproducibility, which can simulate the pathogenesis and clinical characteristics of human ITP. However, as analyzed by the existing literature, there is only the disease model of ITP, but no disease-syndrome combined model of ITP.<sup>7</sup> In order to verify this assumption, we established the differentiation criteria for disease-syndrome combined model of ITP according to the pathogenic and clinical characteristics of human ITP, analyzed/summarized the three batches of data related to the ITP model mice replicated through the passive immune modeling method, and judged whether this combined model conformed to the pathogenesis/syndrome characteristics of human ITP. We believe that it may provide reference to treatment of ITP with reinforcing spleen qi to control blood. As shown by the test results, the replicated APS-injected mice model of ITP possessed the following characteristics: (1) Decrease in platelet count, prolonging in coagulation time which had statistically significance compared with those in the normal group of the same test batch ( $P < .01$ ). (2) Decrease in medullary thrombocyto-genous megakaryocyte, which was statistically significant compared with those in the normal group of the same test batch ( $P < .05$ ,  $.01$ ,  $.001$ ). (3) Spontaneous hemorrhage at the APS injection site or other skin of model mice. (4) One week after the modeling, there were low activity, delayed

activity, poor appetite, decrease in food/water intake, loose stool, skin petechia/hemorrhage and spontaneous hemorrhage at the injected sites or other parts and were getting more and more severe. Therefore, according to the differentiation criteria for the disease-syndrome combined model of ITP, the APS-injected mice model of ITP replicated through the passive immune modeling method possessed the similar pathogenesis/syndrome characteristics of human ITP.

The APS-injected mouse model of ITP replicated through the passive immune modeling method featured various advantages, e.g. high technological maturity, simple operation, good reproducibility, few interfering factors and lower cost.<sup>8,9</sup> However, the establishment of the disease-syndrome combined animal model of ITP without additional conditions is still under exploration. In the past, we attempted to establish a disease-syndrome combined animal model of ITP with the syndrome of blood stasis due to qi deficiency, whose accuracy should still be further evaluated due to difficult selection of blood stasis indices and insufficient evidence of blood stasis.<sup>10</sup> This study specified both the indices for the disease model of ITP and the indices for syndrome transformation. As verified by summarizing and analyzing the various test data according to the differentiation criteria for the disease-syndrome combined model, the objective indices for the APS-injected mouse model of ITP replicated through the passive immune modeling method not only conformed to the pathogenesis characteristics of human ITP, but also the external signs of TCM syndrome for this model were highly correlated to human ITP, which proves that the disease-syndrome combined animal model similar to the pathogenesis of human ITP could be replicated without additional conditions. Meanwhile, as shown by analyzing the continuous dynamic observation results during the test, on Day 3 after injection of APS, a dense scarlet bleeding point appeared on the hypodermis and injection site on the model mice, the subcutaneous petechia merged into flake; and the hemorrhage gradually turned from light-red to dark-red about 3–4 days later. During such transient change, the model mice still had the following obvious signs: low activity, delayed activity, poor appetite, decrease in food/water intake, weight loss and decrease in swimming duration. This phenomenon could not be rationally explained as the syndrome of bleeding due to blood-heat, and should be analyzed through further tests.

According to above results and conclusion, the following issues might require further discussion and improvement.

(1) There is no generally-accepted differentiation criteria for the disease-syndrome combined animal model of ITP, thus the accuracy of diagnostic criteria for syndromes in this study still requires further modification and improvement. (2) APS is an exogenous antibody, and does not originate from the study subject. After withdrawal of APS, the low level of peripheral platelet count maintains for a shorter time in the model mice, thus requiring further tests to verify whether this model is the best for preventive medication.<sup>7</sup> (3) Although every index in this study has the positive results (e.g. decrease in peripheral platelet count, decrease in medullary thrombocytopoietic megakaryocyte and hemorrhage of various degrees) and possesses the similar clinical syndrome/manifestations of human ITP, more test evidences should still be required to determine whether other modeling methods possess the above characteristics.<sup>11,12</sup> (4) Further studies are still needed to verify the clinical significance and expression of platelet membrane glycoprotein IIa/IIIb in this model (as the most convincing anti-platelet antibody). Therefore, in future studies, the animal model of ITP should be replicated in the larger animal (i.e. guinea-pig or rabbit), so as to ensure a sufficient blood specimen for index determination.

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